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(54) Title: PREPARATION AND USE OF IMMUNOCONJUGATES

(57) Abstract

The present invention relates to immunoconjugates comprising an antibody fragment which is covalently bound to a diagnostic or therapeutic principle through a carbohydrate moiety at about position 18 in the light chain variable region of the antibody fragment. The invention also relates to immunoconjugates comprising an antibody moiety that is an intact antibody containing a glycosylation site at about position 18 in the light chain variable domain which has been introduced into the antibody by mutating the nucleotide sequence encoding the light chain. The resultant immunoconjugates retain the immunoreactivity of the antibody fragment or intact antibody, and target the diagnostic or therapeutic principle to a target tissue where the diagnostic or therapeutic effect is realized. Thus, the invention contemplates the use of such immunoconjugates for diagnosis and immunotherapy. The invention further relates to methods for preparing such immunoconjugates.

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PREPARATION AND USE OF IMMUNOCONJUGATES

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention is directed to novel immunoconjugates that are useful for diagnosis and therapy. In particular, this invention is directed to immunoconjugates comprising an antibody fragment which is covalently bound to a diagnostic or therapeutic principle through a carbohydrate moiety in the light chain variable region of the antibody fragment. This invention is also directed to immunoconjugates comprising an antibody moiety that is an intact antibody containing a glycosylation site in the light chain variable domain which has been introduced into the antibody by mutating the nucleotide sequence encoding the light chain. This invention is further directed to methods for preparing such immunoconjugates. This invention also is directed to methods of diagnosis and therapy using such immunoconjugates.

2. Related Art

Monoclonal antibodies can be conjugated to a variety of agents to form immunoconjugates for use in diagnosis and therapy. These agents include chelates, which allow the immunoconjugate to form a stable bond with radioisotopes, and cytotoxic agents such as toxins and chemotherapy drugs. For example, cytotoxic agents that normally would be too toxic to patients when administered in a systemic fashion can be coupled to anti-cancer antibodies in such a manner that their toxic effects become directed only to the tumor cells bearing the target antigens. The diagnostic or therapeutic efficacy of immunoconjugates depends upon several factors. Among these factors, the molar ratio of the diagnostic or therapeutic principle to antibody and the antibody

binding activity of the immunoconjugate are of major concern.

Researchers have found that the maximum number of diagnostic or therapeutic principles that can be directly linked to an antibody is limited by the number of modifiable sites on the antibody molecule and the loss of immunoreactivity of the antibody. For example, Kulkarni *et al.*, *Cancer Research* 41:2700-2706 (1981), have reported that there is a limit to the number of drug molecules that can be incorporated into an antibody without significantly decreasing antigen-binding activity. Kulkarni *et al.*, found that the highest incorporation obtained for methotrexate was about ten methotrexate molecules per molecule of antibody, and that attempts to increase the drug-antibody molar ratio over about ten decreased the yield of immunoconjugate and damaged antibody activity. Kanellos *et al.*, *JNCI* 75:319-329 (1985), have reported similar results.

In order to achieve a high substitution level of drug-immunoconjugate without significantly impairing antigen-binding activity, researchers have investigated the use of a water-soluble polymeric molecule as an intermediary for the indirect conjugation of the drug. Such polymers include oxidized dextran (Arnon *et al.*, *Immunol. Rev.* 62:5-27 (1982)), poly-glutamic acid (Greenfield *et al.*, *Antibody Immunoconjugates and Radiopharmaceuticals* 2:201-216 (1989)), human serum albumin (Baldwin *et al.*, *NCI Monographs* 3:95-99 (1987)), and carboxymethyldextran (Schechter *et al.*, *Cancer Immunol. Immunother.* 25:225-230 (1987)).

Shih *et al.*, *Int. J. Cancer* 41:832-839 (1988), have described a site-specific linking method in which methotrexate was linked to the carbohydrate moiety in the constant, or "Fc," region of an antibody via amino-dextran, resulting in an immunoconjugate with high substitution ratio and retention of immunoreactivity. More recently, Shih *et al.*, *Int. J. Cancer* 46:1101-1106 (1990), demonstrated the efficacy of an immunoconjugate

comprising 5-fluorouridine conjugated via amino-dextran to the carbohydrate moiety in the Fc region of a monoclonal antibody. In both studies, Shih *et al.* found that the immunoconjugate contained approximately 30-50 molecules of drug per molecule of immunoglobulin. Thus, indirect conjugation of a diagnostic or therapeutic principle to a carbohydrate moiety in the Fc region of an antibody provides a means to obtain immunoconjugates with functional antigen binding activity and a high substitution level.

An advantage of using the carbohydrate moiety in the Fc region as a site-specific attachment site is that antibodies of all subtypes typically contain a glycosylated Fc region. In general, antibody molecules are glycosylated in their Fc regions at characteristic positions according to their isotype. For example, carbohydrate is typically present at amino acid 297 in the C_H2 domain in the Fc region of IgG molecules. Conjugating a diagnostic or therapeutic principle to the carbohydrate group at this position, which is far away from the antigen binding site, should produce a minimal effect on the immunoreactivity of the resultant immunoconjugate, as demonstrated by Shih *et al.*

However, a disadvantage of using the carbohydrate moiety in the Fc region as an attachment site is that the entire antibody is required for the immunoconjugate. The use of antibody fragments, particularly Fab, Fab' and $F(ab')_2$ provide an advantage over the use of an entire antibody because such fragments are better able to diffuse out of capillaries and into target tissues. For example, see Brown, "Clinical Use of Monoclonal Antibodies," in BIOTECHNOLOGY AND PHARMACY, Pezzuto *et al.*, eds. Chapman & Hall, pp.227-249 (1993). Moreover, antibody fragments will clear from blood and normal tissues more readily than an entire antibody. For example, intact murine IgG has a blood half-life of approximately 30 hours, while $F(ab')_2$ and Fab/Fab' have

half-lives of approximately 20 hours and 2 hours, respectively. *Id.* Thus, it is advantageous to use antibody fragments for constructing immunoconjugates. Antibody fragments are particularly advantageous in radioimmunotherapy and radioimmunodiagnosis applications in which the exposure of normal tissues to radioisotopes must be minimized.

Antibody variable regions occasionally contain carbohydrate groups which provide potential attachment sites for the preparation of immunoconjugates from antibody fragments. For example, asparagine-linked carbohydrate acceptor sequences have been identified in approximately 15-25% of murine variable regions. Kabat *et al.* SEQUENCES OF PROTEINS OF IMMUNOLOGICAL INTEREST, 5th ed. U.S. Department of Health and Human Services (1990). In the case of the anti-dextran family of antibodies, glycosylation sites reside in the complementarity-determining regions (CDRs), particularly CDR2, of the heavy chain variable regions. *Id.* The presence of Asn-linked carbohydrates in the CDRs of these antibodies appeared to enhance antigen binding. Wallick *et al.*, *J. Exp. Med. 168*:1099-1109 (1988); Wright *et al.*, *EMBO J. 10*:2717-2723 (1991). However, introduction of additional carbohydrate attachment sites in CDR2 by site-directed mutagenesis resulted in either the enhancement or reduction of affinity for antigen, depending on the position where the glycosylation site was introduced. Wright *et al.*, *supra*. Thus, the feasibility of attaching a diagnostic or therapeutic principle to a carbohydrate moiety in the VH CDR region is uncertain.

Studies by the present inventors on carbohydrate conjugation demonstrated a high conjugation efficiency with the IgG antibody, LL2, which is a murine monoclonal antibody described by Pawlak-Byczkowska *et al.* (*Cancer Res. 49*:4568-4577 (1989)) and Goldenberg *et al.* (*J. Clin. Oncol. 9*:548 (1991)). Analysis of LL2 conjugates using sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions indicated the

existence of a glycosylation site in the light chain variable (VL) region of the LL2 antibody. After cloning the VL region of LL2, an Asn-linked glycosylation site at position 18-20 of the framework-1 (FR1) sequence of the VL region was found.

These studies suggested a possible preferential conjugation at a carbohydrate moiety within the VL region. This unexpected finding may be explained by an improved accessibility in the VL region. We used site-directed mutagenesis to remove the Asn-linked glycosylation site and found that the resulting protein exhibited similar immunoreactivity compared with the original antibody. This result is in agreement with the inventors' computer modeling studies which suggested negligible or minimal interaction between the light chain FR1 carbohydrate moiety and the antigen binding site. Thus, these studies indicate that conjugation of a diagnostic or therapeutic principle to a carbohydrate moiety in the FR1 sequence of the VL region provides a means to obtain immunoconjugates of antibody fragments with functional antigen binding activity.

The present invention provides a method for preparing novel immunoconjugates comprising a diagnostic or therapeutic principle which is attached to an intact antibody, or antigen-binding fragment thereof, via a carbohydrate moiety of the light chain variable region.

SUMMARY OF THE INVENTION

The present invention is directed to a mutated recombinant antibody or antibody fragment having a non-natural Asn-glycosylation site at about position 18 of the light chain of said antibody or antibody fragment.

The present invention is also directed to a method for preparing a glycosylated mutated recombinant antibody or antibody fragment, comprising the steps of:

(a) culturing transformed host cells which express and glycosylate a mutated antibody or antibody fragment comprising a mutated light chain and a heavy chain,

said host cells being transformed with an expression vector into which is cloned a mutated DNA molecule encoding a mutated light chain containing a non-natural Asn-glycosylation site at about amino acid position 18; and

(b) recovering expressed and glycosylated mutated antibody or antibody fragment from said cultured host cells.

The present invention is further directed to a soluble immunoconjugate, comprising:

(a) a glycosylated antibody fragment, wherein the antibody fragment is selected from the group consisting of Fab, Fab', $F(ab)_2$, and $F(ab')_2$, and wherein the antibody fragment comprises a light chain variable region and a carbohydrate moiety attached at about amino acid position 18 of the light chain variable region; and

(b) an intermediate conjugate, comprising a polymer carrier having at least one free amine group and a plurality of drug, toxin, chelator, boron addend or detectable label molecules covalently bound to the polymer carrier, wherein the intermediate conjugate is covalently bound through at least one free amine group of the polymer carrier to the carbohydrate moiety of the antibody fragment, and wherein the immunoconjugate retains the immunoreactivity of the antibody fragment.

In addition, the present invention is directed to a soluble immunoconjugate, comprising:

(a) a glycosylated antibody fragment, wherein the antibody fragment is selected from the group consisting of Fab, Fab', $F(ab)_2$, and $F(ab')_2$, and wherein the antibody fragment comprises a light chain variable region and a carbohydrate moiety attached at about amino acid position 18 of the light chain variable region; and

(b) a non-antibody moiety selected from the group consisting of a drug, a toxin, a chelator, a polyethylene glycol, a boron addend and a detectable label molecule, wherein the non-antibody moiety is covalently bound to the carbohydrate moiety of the antibody fragment, and wherein the immunoconjugate retains the immunoreactivity of the antibody fragment.

The present invention is further directed to a soluble immunoconjugate, comprising:

(a) a mutated antibody, wherein the mutated antibody comprises a light chain variable region and a carbohydrate moiety attached at about amino acid position 18 of the light chain variable region; and

(b) a non-antibody moiety selected from the group consisting of a drug, a toxin, a chelator, a polyethylene glycol, a boron addend and a detectable label molecule,

wherein the non-antibody moiety is covalently bound to the carbohydrate moiety of the mutated antibody,

and wherein the immunoconjugate retains the immunoreactivity of the mutated antibody.

The present invention is also directed to a soluble immunoconjugate, comprising:

(a) an antibody component, wherein the antibody component is selected from the group consisting of an Fv and a single chain antibody, and wherein the antibody component comprises a light chain variable region and a carbohydrate moiety attached at about amino acid position 18 of the light chain variable region; and

(b) an intermediate conjugate, comprising a polymer carrier having at least one free amine group and a plurality of drug, toxin, chelator, boron addend or detectable label molecules covalently bound to the polymer carrier,

wherein the intermediate conjugate is covalently bound through at least one free amine group of the polymer carrier to the carbohydrate moiety of the antibody component,

and wherein the immunoconjugate retains the immunoreactivity of the antibody component.

The present invention is further directed to a soluble immunoconjugate, comprising:

(a) an antibody component, wherein the antibody component is selected from the group consisting of an Fv and a single chain antibody, and wherein the antibody component comprises a light chain variable region and a carbohydrate moiety attached at about amino acid position 18 of the light chain variable region; and

(b) a non-antibody component selected from the group consisting of a drug, a toxin, a chelator, a polyethylene glycol, a boron addend and a detectable label molecule,

wherein the non-antibody component is covalently bound to the carbohydrate moiety of the antibody component,

and wherein the immunoconjugate retains the immunoreactivity of the antibody component.

The present invention is also directed to a method for preparing an immunoconjugate, comprising the steps of:

(a) introducing an Asn-glycosylation site at about position 18 of the light chain of an antibody by mutating the nucleotide sequence of a DNA molecule encoding the light chain;

(b) cloning the mutated DNA molecule into an expression vector;

(c) transforming host cells with the expression vector, and recovering transformed host cells which express a mutated antibody comprising a mutated light chain and a heavy chain;

(d) culturing the transformed host cells and recovering the mutated antibody from the cultured host cells;

(e) preparing an antibody fragment from the recovered antibody, wherein the antibody fragment is selected from the group consisting of Fab, Fab', $F(ab)_2$, and $F(ab')_2$, and wherein the antibody fragment contains a carbohydrate moiety in the mutated light chain of the antibody fragment; and

(f) covalently binding an intermediate conjugate to the carbohydrate moiety of the antibody fragment, wherein the intermediate conjugate comprises a polymer carrier having at least one free amine group and a plurality of drug, toxin, chelator, boron addend or detectable label molecules covalently bound to the polymer carrier, wherein the intermediate conjugate is covalently bound through at least one free amine group of the polymer carrier to the carbohydrate moiety of the antibody fragment, and wherein the immunoconjugate retains the immunoreactivity of the antibody fragment.

The present invention is also directed to a method for diagnosing the presence of a disease in a mammal, comprising the steps of:

(a) preparing an immunoconjugate comprising a detectable label and an antibody fragment having a carbohydrate moiety attached at about position 18 of the light chain of the antibody fragment, wherein the detectable label is conjugated to the carbohydrate moiety of the antibody fragment, and wherein the antibody fragment is capable of binding to an antigen which is associated with the disease;

(b) administering a composition comprising the immunoconjugate and a pharmaceutically acceptable carrier to the mammal; and

(c) using *in vivo* imaging to detect the presence of the immunoconjugate at disease sites.

The present invention is further directed to a method for treating a disease in a mammal, comprising the steps of:

(a) preparing an immunoconjugate comprising an antibody fragment having a carbohydrate moiety attached at about position 18 of the light chain of the antibody fragment and a non-antibody moiety selected from the group consisting of a drug, a toxin, a chelator, a boron addend and a radioisotope, wherein the non-antibody moiety is covalently bound to the carbohydrate moiety of the antibody fragment, and wherein the antibody fragment is capable of binding to an antigen which is associated with the disease; and

(b) administering a composition comprising the immunoconjugate and a pharmaceutically acceptable carrier to the mammal.

Also included in the present invention are improved methods of *in vitro* immunoassay and *in situ* detection of antigen in histological specimens using the immunoconjugates of the invention.

There are also provided suitable polymer carriers, chelates, detectable label molecules, and linking moieties suitable for use in preparing the immunoconjugates of the invention.

DETAILED DESCRIPTION

1. Overview

This invention is directed to immunoconjugates comprising an intact antibody, or antigen-binding fragment thereof, which is covalently bound to a diagnostic or therapeutic principle through a carbohydrate moiety in the light chain variable region of the antibody moiety. This invention further relates to

methods for preparing such immunoconjugates. The invention also contemplates the use of such immunoconjugates for diagnosis and immunotherapy.

2. Definitions

In the description that follows, a number of terms are utilized extensively. Definitions are herein provided to facilitate understanding of the invention.

Antibody. As used herein, "antibody" includes monoclonal antibodies, such as murine, chimeric, or humanized antibodies, as well as antigen-binding fragments thereof. Such fragments include Fab, Fab', $F(ab)_2$, and $F(ab')_2$, which lack the Fc fragment of an intact antibody. Such fragments also include isolated fragments consisting of the light chain variable region, "Fv" fragments consisting of the variable regions of the heavy and light chains, and recombinant single chain polypeptide molecules in which light and heavy variable regions are connected by a peptide linker.

Mutated Antibody. As used herein, a mutated antibody is an intact antibody, or antigen-binding fragment thereof, having an Asn-linked glycosylation site at about amino acid position 18 in the light chain, which has been introduced into the light chain by altering the corresponding nucleotide sequence. Methods of mutating the nucleotide sequence encoding a light chain include the polymerase chain reaction, site-directed mutagenesis, and gene synthesis using the polymerase chain reaction with synthetic DNA oligomers.

Diagnostic or Therapeutic Principle. As used herein, a diagnostic or therapeutic principle is a molecule or atom which is conjugated to an antibody to produce an immunoconjugate which is useful for diagnosis and for therapy. Examples of diagnostic or therapeutic principles include drugs, toxins, chelators, boron compounds, and detectable labels.

Immunoconjugate. As used herein, an immunoconjugate is a molecule comprising an antibody and a diagnostic or

therapeutic principle. An immunoconjugate retains the immunoreactivity of the antibody, i.e., the antibody moiety has roughly the same, or only slightly reduced, ability to bind the antigen after conjugation as before conjugation.

Structural gene. A DNA sequence that is transcribed into messenger RNA (mRNA) which is then translated into a sequence of amino acids characteristic of a specific polypeptide.

Promoter. A DNA sequence which directs the transcription of a structural gene to produce mRNA. Typically, a promoter is located in the 5′ region of a gene, proximal to the start codon of a structural gene. If a promoter is an inducible promoter, then the rate of transcription increases in response to an inducing agent. In contrast, the rate of transcription is not regulated by an inducing agent if the promoter is a constitutive promoter.

Enhancer. A promoter element. An enhancer can increase the efficiency with which a particular gene is transcribed into mRNA irrespective of the distance or orientation of the enhancer relative to the start site of transcription.

Complementary DNA (cDNA). Complementary DNA is a single-stranded DNA molecule that is formed from an mRNA template by the enzyme reverse transcriptase. Typically, a primer complementary to portions of mRNA is employed for the initiation of reverse transcription. Those skilled in the art also use the term "cDNA" to refer to a double-stranded DNA molecule consisting of such a single-stranded DNA molecule and its complement.

Expression. Expression is the process by which a polypeptide is produced from a structural gene. The process involves transcription of the gene into mRNA and the translation of such mRNA into polypeptide(s).

Cloning vector. A DNA molecule, such as a plasmid, cosmid, or bacteriophage, which has the capability of replicating autonomously in a host cell and which is used

to transform cells for gene manipulation. Cloning vectors typically contain one or a small number of restriction endonuclease recognition sites at which foreign DNA sequences may be inserted in a determinable fashion without loss of an essential biological function of the vector, as well as a marker gene which is suitable for use in the identification and selection of cells transformed with the cloning vector. Marker genes typically include genes that provide tetracycline resistance or ampicillin resistance.

Expression vector. A DNA molecule comprising a cloned structural gene encoding a foreign protein which provides the expression of the foreign protein in a recombinant host. Typically, the expression of the cloned gene is placed under the control of (i.e., operably linked to) certain regulatory sequences such as promoter and enhancer sequences. Promoter sequences may be either constitutive or inducible.

Recombinant Host. A recombinant host may be any prokaryotic or eukaryotic cell which contains either a cloning vector or expression vector. This term is also meant to include those prokaryotic or eukaryotic cells that have been genetically engineered to contain the cloned gene(s) in the chromosome or genome of the host cell. For examples of suitable hosts, see Sambrook *et al.*, MOLECULAR CLONING: A LABORATORY MANUAL, Second Edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1989).

3. Methods for Introducing an Asn-glycosylation Site in an Antibody Light Chain by Mutating the DNA Sequence Encoding the Protein

A. Antibody Structure and Asn-linked Glycosylation

Antibody molecules are composed of two identical copies of heavy chains and light chains, which are

covalently interconnected by disulfide bonds. For a general discussion, see Schultz et *al.*, "Proteins II: Structure-Function Relationship of Protein Families," in TEXTBOOK OF BIOCHEMISTRY WITH CLINICAL CORRELATIONS, 3rd Ed., T.M. Devlin (ed.), Wiley & Sons, pp. 92-134 (1992); Turner et *al.*, "Antigen Receptor Molecules," in IMMUNOLOGY, 3rd Ed., Roitt et *al.* (eds.), Mosby, pp. 4.1-4.20 (1993). In the most common type of antibody, IgG, the two heavy chains each have approximately 440 amino acids, while the two light chains each have about 220 amino acids. The carboxyl-terminal one-half of light chains and the carboxyl-terminal three-quarters of heavy chains are highly conserved in amino acid sequence among antibodies with different antigen specificities. These conserved regions in the light and heavy chains are termed "constant regions" and are designated as CL and CH, respectively. The CH regions determine whether a particular antibody belongs to the antibody class IgG, IgA, IgD, IgE, or IgM. The CH regions within a class of antibodies are homologous but differ significantly from the amino acid sequence of the CH regions of other antibody classes.

In contrast, the amino acid sequences of the amino-terminal one-half of the light chains and the amino-terminal of one-quarter of the heavy chains are highly variable among antibodies with different antigen specificities. Particular regions within these variable segments are "hypervariable" and have been designated as "complementarity determining regions" (CDRs) because these regions form the antigen binding site (ABS) that is complementary to the topology of the antigen structure.

Each heavy chain is associated with a light chain such that the amino-terminal ends of both chains are near each other and comprise an antigen binding site. Proteolytic cleavage can be used to fragment an antibody into small, functional units. For example, proteolytic cleavage of an IgG molecule with papain results in the

cleavage of the antibody in the hinge peptide of each heavy chain. One product of papain digestion is the carboxyl-terminal one-half of the heavy chains which are bound covalently in a "crystallizable fragment" (Fc). The Fc fragment does not bind antigen. The other cleavage products are identical and consist of an amino-terminal segment of a heavy chain which is associated with an entire light chain. These amino-terminal, or "antigen binding fragments" (Fab) can bind antigen with an affinity similar to that of the intact antibody molecule.

The object of the present invention is to covalently attach a diagnostic or therapeutic principle to an Asn-linked carbohydrate moiety of the light chain variable region of an intact antibody, or antigen-binding fragment thereof. Asn-linked glycosylation, also referred to as "N-linked glycosylation," is a form of glycosylation in which sugar residues are linked through the amide nitrogen of asparagine residues. Intracellular biosynthesis of Asn-linked oligosaccharides occurs in both the lumen of the endoplasmic reticulum and following transport of the protein to the Golgi apparatus. Asn-linked glycosylation occurs at the glycosylation sequence: Asn-X-Thr/Ser, where X may be any amino acid except proline or aspartic acid. Thus, there are 36 possible sequences of three amino acids which code for Asn-linked glycosylation. Considering the degeneracy of the genetic code, there are over a thousand possible nucleotide sequences which encode the glycosylation signal sequences.

B. Mutagenesis

The particular nucleotide sequence which is used to introduce an Asn-linked glycosylation sequence into positions 18-20 will depend upon the naturally-occurring nucleotide sequence. As described below, the introduction of an Asn-linked glycosylation site into the PKAPPA(11)24 protein can be achieved by an alteration of

codon 18 from AGG to AAC. Such a mutation of the nucleotide sequence can be accomplished by methods well-known to those in the art.

For example, an Asn-linked glycosylation site can be introduced at positions 18-20 using oligonucleotide-directed mutagenesis and a cloned antibody light chain. In this procedure, a single-stranded DNA template containing the antibody light chain sequence is prepared from a *dut⁻ ung⁻* strain of *E. coli* in order to produce a DNA molecule containing a small number of uracil residues in place of thymidine. Such a DNA template can be obtained by M13 cloning or by *in vitro* transcription using an SP6 promoter. See, for example, Ausubel *et al.* (eds.), CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley & Sons (1987). An oligonucleotide that contains the mutated sequence is synthesized using well-known methods. *Id.* The oligonucleotide is annealed to the single-stranded template, and T4 DNA polymerase and T4 DNA ligase are used to produce a double-stranded DNA molecule. Transformation of wild-type (*dut⁺ ung⁺*) *E. coli* cells with the double-stranded DNA molecule provides an efficient recovery of mutated DNA.

Detailed protocols for oligonucleotide-directed mutagenesis and related techniques for mutagenesis of cloned DNA are well-known. For example, see Ausubel *et al.*, *supra*; Sambrook *et al.*, *supra*.

Alternatively, an Asn-linked glycosylation site can be introduced into an antibody light chain using an oligonucleotide containing the desired mutation as a primer and DNA clones of the variable regions for the antibody light chain, or by using RNA from cells that produce the antibody of interest as a template. Such techniques include, for example, the polymerase chain reaction, as illustrated in Example 1. Also see, Huse, "Combinatorial Antibody Expression Libraries in Filamentous Phage," in ANTIBODY ENGINEERING: A PRACTICAL GUIDE, C. Borrebaeck (ed.), W.H. Freeman and Company, pp. 103-120 (1992). Site-directed mutagenesis can be

performed, for example, using the TRANSFORMER™ Site-Directed Mutagenesis Kit (Clontech; Palo Alto, CA) according the manufacturer's instructions.

Alternatively, a glycosylation site can be introduced into an immunoglobulin light chain by synthesizing a light chain gene with mutually priming oligonucleotides in which one of the oligonucleotides contains the desired mutation. Techniques for the construction of large synthetic genes are well known to those in the art. See, for example, Uhlmann, *Gene 71*:29-40 (1988); Wosnick *et al.*, *Gene 60*:115-127 (1988); Ausubel *et al.*, *supra*.

In summary, an Asn-linked glycosylation site can be introduced at about amino acid position 18 in the light chain of any antibody if two requirements are met. First, the nucleotide sequence surrounding and including the codons for amino acid positions 18-20 of the light chain of the antibody of interest must be available in order to design a complementary oligonucleotide containing the desired mutation. Second, there must be access to either cloned antibody DNA or cells that produce the antibody of interest. Given these two restrictions, the present invention encompasses immunoconjugates comprising murine, humanized, or chimeric antibodies, wherein a diagnostic or therapeutic principle is attached to the antibody component via a carbohydrate moiety located at about amino acid position 18 of the light chain variable region. Such antibodies include intact antibodies and the antigen-binding fragments, Fab, Fab', $F(ab)_2$, and $F(ab')_2$.

Moreover, the present invention contemplates the production of immunoconjugates comprising Fv fragments or single chain antibodies. As discussed above, Fv fragments comprise a non-covalent association of heavy and light chain variable regions. In contrast, single-chain antibodies comprise heavy and light polypeptide chains from the variable region of a given antibody which are connected by a peptide linker. See, for example, Bird *et al.*, *Science 242*:423-426 (1988); Ladner *et al.*,

U.S. Patent No. 4,946,778; and Pack *et al.*, *Bio/Technology* 11:1271-1277 (1993).

Generally, Fv fragments and single chain antibodies lack a site for attaching certain diagnostic or therapeutic principles, such as radiometals. However, the introduction of an Asn-linked glycosylation site into a light chain variable region of an Fv fragment or single chain antibody provides a carbohydrate moiety for the attachment of a variety of diagnostic or therapeutic principles, as described below. Although Fv fragments and single chain antibodies are typically produced by prokaryotic host cells, eukaryotic host cells are preferred host cells. In particular, insect cells, yeast cells, and mammalian cells are preferred eukaryotic hosts. Mammalian cells are the most preferred host cells.

Although the present invention provides a method for introducing an Asn-linked glycosylation site at about amino acid position 18-20 of the light chain variable region, it will be understood that the present invention is not so limited. It will occur to those of ordinary skill in the art that it is possible to introduce glycosylation sites at alternative positions of the light chain variable region, or even in the heavy chain variable region. Immunoconjugates of the present invention can be prepared using intact antibodies, antibody fragments, or single chain antibodies which contain a carbohydrate moiety attached at such an alternate glycosylation site as long as the mutated antibodies or fragments retain antigen-binding activity. Suitable alternative glycosylation sites can be identified using molecular modeling techniques that are well-known to those of skill in the art. See, for example, Lesk *et al.*, "Antibody Structure and Structural Predictions Useful in Guiding Antibody Engineering," in ANTIBODY ENGINEERING: A PRACTICAL GUIDE, C. Borrebaeck (ed.), W.H. Freeman and Company, pp. 1-38 (1992);

Cheetham, "Engineering Antibody Affinity" *Id.* at pp. 39-67.

4. Methods for Expressing and Isolating the Protein Product of a Mutated Antibody DNA Sequence

A. Methods for Expressing a Mutated Antibody

After mutating the nucleotide sequence, mutated DNA is inserted into a cloning vector for further analysis, such as confirmation of the DNA sequence, as illustrated in Example 1. To express the polypeptide encoded by the mutated DNA sequence, the DNA sequence must be operably linked to regulatory sequences controlling transcriptional expression in an expression vector and then, introduced into either a prokaryotic or eukaryotic host cell. In addition to transcriptional regulatory sequences, such as promoters and enhancers, expression vectors include translational regulatory sequences and a marker gene which is suitable for selection of cells that carry the expression vector.

Suitable promoters for expression in a prokaryotic host can be repressible, constitutive, or inducible. Suitable promoters are well-known to those of skill in the art and include promoters capable of recognizing the T4, T3, Sp6 and T7 polymerases, the P_R and P_L promoters of bacteriophage lambda, the trp, recA, heat shock, and lacZ promoters of *E. coli*, the α-amylase and the σ^N-specific promoters of *B. subtilis*, the promoters of the bacteriophages of *Bacillus*, *Streptomyces* promoters, the *int* promoter of bacteriophage lambda, the *bla* promoter of the β-lactamase gene of pBR322, and the CAT promoter of the chloramphenicol acetyl transferase gene. Prokaryotic promoters are reviewed by Glick, *J. Ind. Microbiol.* 1:277-282 (1987); Watson et *al.*, MOLECULAR BIOLOGY OF THE GENE, 4th Ed., Benjamin Cummins (1987); Ausubel et *al.*, *supra*, and Sambrook et *al.*, *supra*.

An especially preferred prokaryotic host is *E. coli*. Preferred strains of *E. coli* include Y1088, Y1089, CSH18, ER1451, and ER1647 (see, for example, Brown (Ed.), MOLECULAR BIOLOGY LABFAX, Academic Press (1991)). An alternative preferred host is *Bacillus subtilus*, including such strains as BR151, YB886, MI119, MI120, and B170 (see, for example, Hardy, "Bacillus Cloning Methods," in DNA CLONING: A PRACTICAL APPROACH, Glover (Ed.), IRL Press (1985)).

Methods for producing antibody fragments in *E. coli* are well-known to those in the art. See, for example, Huse, "Combinatorial Antibody Expression Libraries in Filamentous Phage," in ANTIBODY ENGINEERING: A PRACTICAL GUIDE, C. Borrebaeck (Ed.), W.H. Freeman and Company, pp. 103-120 (1992); Ward, "Expression and Purification of Antibody Fragments Using *Escherichia coli* as a Host," *Id.* at pp. 121-138 (1992). Those skilled in the art also know methods for producing in *E. coli* Fv fragments, which consist of variable regions of heavy and light chains. *Id.* Also, see Whitlow *et al.*, "Single-Chain Fv Proteins and their Fusion Proteins," in NEW TECHNIQUES IN ANTIBODY GENERATION, *Methods 2(2)* (1991).

Moreover, expression systems for cloning antibodies in prokaryotic cells are commercially available. For example, the IMMUNO ZAP™ Cloning and Expression System (Stratagene Cloning Systems; La Jolla, CA) provides vectors for the expression of antibody light and heavy chains in *E. coli*.

Since the expression of a mutated DNA sequence in prokaryotic cells will require subsequent *in vitro* glycosylation, the present invention preferably encompasses the expression of a mutated DNA sequence in eukaryotic cells, and especially mammalian, insect, and yeast cells. Especially preferred eukaryotic hosts are mammalian cells. Mammalian cells provide post-translational modifications to the cloned polypeptide including proper folding and glycosylation. For example, such mammalian host cells include COS-7 cells (ATCC CRL

1651), non-secreting myeloma cells (SP2/0-AG14; ATCC CRL 1581), Chinese hamster ovary cells (CHO-K1; ATCC CCL 61), rat pituitary cells (GH_1; ATCC CCL 82), HeLa S3 cells (ATCC CCL 2.2), and rat hepatoma cells (H-4-II-E; ATCC CRL 1548).

For a mammalian host, the transcriptional and translational regulatory signals may be derived from viral sources, such as adenovirus, bovine papilloma virus, and simian virus. In addition, promoters from mammalian expression products, such as actin, collagen, or myosin, can be employed. Alternatively, a prokaryotic promoter (such as the bacteriophage T3 RNA polymerase promoter) can be employed, wherein the prokaryotic promoter is regulated by a eukaryotic promoter (for example, *see* Zhou *et al.*, *Mol. Cell. Biol. 10*:4529-4537 (1990); Kaufman *et al.*, *Nucl. Acids Res. 19*:4485-4490 (1991)). Transcriptional initiation regulatory signals may be selected which allow for repression or activation, so that expression of the genes can be modulated.

In general, eukaryotic regulatory regions will include a promoter region sufficient to direct the initiation of RNA synthesis. Such eukaryotic promoters include the promoter of the mouse metallothionein I gene (Hamer *et al.*, *J. Mol. Appl. Gen. 1*:273-288 (1982)); the TK promoter of Herpes virus (McKnight, *Cell 31*:355-365 (1982)); the SV40 early promoter (Benoist *et al.*, *Nature (London) 290*:304-310 (1981)); the Rous sarcoma virus promoter (Gorman *et al.*, *supra*); the cytomegalovirus promoter (Foecking *et al.*, *Gene 45*:101 (1980)); the yeast *gal4* gene promoter (Johnston, *et al.*, *Proc. Natl. Acad. Sci. (USA) 79*:6971-6975 (1982); Silver, *et al.*, *Proc. Natl. Acad. Sci. (USA) 81*:5951-5955 (1984)); and the IgG promoter (Orlandi *et al.*, *Proc. Natl. Acad. Sci. USA 86*:3833-3837 (1989)).

Strong regulatory sequences are the most preferred regulatory sequences of the present invention. Examples of such preferred regulatory sequences include the SV40 promoter-enhancer (Gorman, "High Efficiency Gene Transfer

into Mammalian cells," in DNA CLONING: A PRACTICAL APPROACH, Volume II, Glover (Ed.), IRL Press pp. 143-190 (1985)), the hCMV-MIE promoter-enhancer (Bebbington *et al.*, *Bio/Technology* *10*:169-175 (1992)), and antibody heavy chain promoter (Orlandi *et al.*, *Proc. Natl. Acad. Sci. USA* *86*:3833-3837 (1989)). Also preferred are the kappa chain enhancer for the expression of the light chain and the IgH enhancer (Gillies, "Design of Expression Vectors and Mammalian Cell Systems Suitable for Engineered Antibodies," in ANTIBODY ENGINEERING: A PRACTICAL GUIDE, C. Borrebaeck (Ed.), W.H. Freeman and Company, pp. 139-157 (1992); Orlandi *et al.*, *supra*).

The mutated antibody-encoding sequence and an operably linked promoter may be introduced into eukaryotic cells as a non-replicating DNA molecule, which may either be a linear molecule or, more preferably, a closed covalent circular molecule. Since such molecules are incapable of autonomous replication, the expression of the protein may occur through the transient expression of the introduced sequence. Preferably, permanent expression occurs through the integration of the introduced sequence into the host chromosome.

Preferably, the introduced sequence will be incorporated into a plasmid or viral vector that is capable of autonomous replication in the recipient host. Several possible vector systems are available for this purpose. One class of vectors utilize DNA elements which provide autonomously replicating extra-chromosomal plasmids, derived from animal viruses such as bovine papilloma virus, polyoma virus, adenovirus, or SV40 virus. A second class of vectors relies upon the integration of the desired genomic or cDNA sequences into the host chromosome. Additional elements may also be needed for optimal synthesis of mRNA. These elements may include splice signals, as well as transcription promoters, enhancers, and termination signals. The cDNA expression vectors incorporating such elements include

those described by Okayama, *Mol. Cell. Biol.* 3:280 (1983), Sambrook *et al.*, *supra*, Ausubel *et al.*, *supra*, Bebbington *et al.*, *supra*, Orlandi *et al.*, *supra*, and Fouser *et al.*, *Bio/Technology* 10:1121-1127 (1992); Gillies, *supra*. Genomic DNA expression vectors which include intron sequences are described by Orlandi *et al.*, *supra*.. Also, see generally, Lerner *et al.* (Eds.), NEW TECHNIQUES IN ANTIBODY GENERATION, *Methods 2(2)* (1991).

In order to obtain mammalian cells that express intact antibody, the expression vector comprising the mutated antibody light chain can be co-transfected into mammalian cells with an antibody heavy chain expression vector. See, for example, Orlandi *et al.*, *supra*. Alternatively, mammalian cells containing a heavy chain expression vector can be transfected with an expression vector comprising the mutated antibody light chain, and mammalian cells containing an expression vector comprising a mutated light chain can be transfected with a heavy chain expression vector. Moreover, mammalian cells can be transfected with a single expression vector comprising DNA fragments that encode the mutated antibody light chain, as well as DNA fragments that encode antibody heavy chain. See, for example, Gillies, *supra*; Bebbington *et al.*, *supra*. Any of these approaches will produce transfected cells that express whole antibody molecules which have the mutated antibody light chain. Standard transfection techniques are well known in the art. See, for example, Sambrook *et al.*, *supra*; Ausubel *et al.*, *supra*.

B. Methods for Isolating a Mutated Antibody from Transfected Cells

Transfected cells that carry the expression vector are selected using the appropriate drug. For example, G418 can be used to select transfected cells carrying an expression vector having the aminoglycoside phosphotransferase gene. Southern *et al.*, *J. Mol. Appl. Gen.* 1:327-341 (1982). Alternatively, hygromycin-B can

be used to select transfected cells carrying an expression vector having the hygromycin-B-phosphotransferase gene. Palmer *et al., Proc. Natl. Acad. Sci. USA* 84:1055-1059 (1987). Alternatively, aminopterin and mycophenolic acid can be used to select transfected cells carrying an expression vector having the xanthine-guanine phosphoribosyltransferase gene. Mulligan *et al., Proc. Natl. Acad. Sci. USA* 78:2072-2076 (1981).

Transfected cells that produce the mutated antibody can be identified using a variety of methods. For example, any immunodetection assay can be used to identify such "transfectomas." Example 1 provides an illustration of the use of an enzyme-linked immunosorbent assay (ELISA) for such a purpose.

After transfectomas have been identified, the cells are cultured and antibodies are isolated from culture supernatants. Isolation techniques include affinity chromatography with Protein-A Sepharose (for intact antibodies), size-exclusion chromatography, and ion-exchange chromatography. For example, see Coligan *et al.* (eds.), CURRENT PROTOCOLS IN IMMUNOLOGY, John Wiley & Sons (1991), for detailed protocols.

5. Methods for Preparing Immunoconjugates

A. Preparation of Antibody Fragments

The present invention contemplates the preparation of immunoconjugates from intact mutated antibodies or from antigen-binding antibody fragments. Antibody fragments can be obtained from transfectomas, by proteolytic cleavage of intact mutant antibodies produced by transfectomas, or by proteolytic cleavage of intact antibodies that have naturally-occurring Asn-linked glycosylation sites at position 18-20 of the light chain.

Antibody fragments can be obtained directly from transfectomas by transfecting cells with a heavy chain structural gene that has been mutated. For example, transfectomas should produce Fab fragments if a stop

codon was inserted following the sequence of the CH1 domain. Alternatively, transfectomas should produce Fab' or $F(ab')_2$ fragments if a stop codon was inserted after the sequence encoding the hinge region of the heavy chain.

Alternatively, antibody fragments can be prepared from intact antibodies using well-known proteolytic techniques. For example, see, Coligan *et al.*, *supra*. As an illustration, Example 2 provides a method to obtain Fab fragments using papain. Moreover, $F(ab')_2$ fragments can be obtained using pepsin digestion of intact antibodies. Divalent fragments can be cleaved to monovalent fragments using conventional disulfide bond reducing agents, e.g., cysteine, dithiothreitol (DTT), and the like.

B. Methods of Conjugation

(i) Indirect conjugation

Immunoconjugates can be prepared by indirectly conjugating a diagnostic or therapeutic principle to an intact antibody, or antigen-binding fragment thereof. Such techniques are described in Shih *et al.*, *Int. J. Cancer* *41*:832-839 (1988); Shih *et al.*, *Int. J. Cancer* *46*:1101-1106 (1990); and Shih *et al.*, U.S. Patent No. 5,057,313. The general method involves reacting an antibody component having an oxidized carbohydrate portion with a carrier polymer that has at least one free amine function and that is loaded with a plurality of drug, toxin, chelator, or boron addends, or with detectable labels. This reaction results in an initial Schiff base (imine) linkage, which can be stabilized by reduction to a secondary amine to form the final conjugate.

The carrier polymer is preferably an aminodextran or polypeptide of at least 50 amino acid residues, although other substantially equivalent polymer carriers can also be used. Preferably, the final immunoconjugate is

soluble in an aqueous solution, such as mammalian serum, for ease of administration and effective targeting for use in diagnosis or therapy. Thus, solubilizing functions on the carrier polymer will enhance the serum solubility of the final immunoconjugate. Solubilizing functions also are important for use of immunoconjugates for *in vitro* immunoassay and *in situ* detection, as described below. In particular, an aminodextran will be preferred.

The process for preparing an immunoconjugate with an aminodextran carrier typically begins with a dextran polymer, advantageously a dextran of average molecular weight of about 10,000 - 100,000. The dextran is reacted with an oxidizing agent to effect a controlled oxidation of a portion of its carbohydrate rings to generate aldehyde groups. The oxidation is conveniently effected with glycolytic chemical reagents such as $NaIO_4$, according to conventional procedures.

The oxidized dextran is then reacted with a polyamine, preferably a diamine, and more preferably, a mono- or polyhydroxy diamine. Suitable amines include ethylene diamine, propylene diamine, or other like polymethylene diamines, diethylene triamine or like polyamines, 1,3-diamino-2-hydroxypropane, or other like hydroxylated diamines or polyamines, and the like. An excess of the amine relative to the aldehyde groups of the dextran is used to insure substantially complete conversion of the aldehyde functions to Schiff base groups.

A reducing agent, such as $NaBH_4$, $NaBH_3CN$ or the like, is used to effect reductive stabilization of the resultant Schiff base intermediate. The resultant adduct can be purified by passage through a conventional sizing column to remove cross-linked dextrans.

Other conventional methods of derivatizing a dextran to introduce amine functions can also be used, e.g., reaction with cyanogen bromide, followed by reaction with a diamine.

The aminodextran is then reacted with a derivative of the particular drug, toxin, chelator, boron addend, or label to be loaded, in an activated form, preferably, a carboxyl-activated derivative, prepared by conventional means, e.g., using dicyclohexylcarbodiimide (DCC) or a water soluble variant thereof, to form an intermediate adduct.

Alternatively, polypeptide toxins such as pokeweed antiviral protein or ricin A-chain, and the like, can be coupled to aminodextran by glutaraldehyde condensation or by reaction of activated carboxyl groups on the protein with amines on the aminodextran.

Chelators for radiometals or magnetic resonance enhancers are well-known in the art. Typical are derivatives of 1,4,7,10-tetraazacyclododecanetetraacetic acid (DOTA), ethylenediaminetetraacetic acid (EDTA), and diethylenetriaminepentaacetic acid (DTPA). These chelators typically have groups on the side chain by which the chelator can be attached to a carrier. Such groups include, e.g., benzylisothiocyanate, by which the DOTA, DTPA or EDTA can be coupled to the amine group of a carrier. Alternatively, carboxyl groups or amine groups on a chelator can be coupled to a carrier by activation or prior derivatization and then coupling, all by well-known means.

Labels such as enzymes, fluorescent compounds, electron transfer agents, and the like can be linked to a carrier by conventional methods well known to the art. These labeled carriers and the immunoconjugates prepared from them can be used for *in vitro* immunoassays and for *in situ* detection, as described below.

Boron addends, such as carboranes, can be attached to antibody components by conventional methods. For example, carboranes can be prepared with carboxyl functions on pendant side chains, as is well known in the art. Attachment of such carboranes to a carrier, e.g., aminodextran, can be achieved by activation of the carboxyl groups of the carboranes and condensation with

amines on the carrier to produce an intermediate conjugate. Such intermediate conjugates are then attached to antibody components to produce therapeutically useful immunoconjugates, as described below.

As an alternative to aminodextran, a polyamidoamine dendrimer may be used as a carrier polymer. Dendrimer molecules of a suitable type can be prepared, for example, by the method of Tomalia *et al., Angew. Chem. Int. Ed. Engl.* **29**: 138-175 (1990). Dendrimers prepared by this method exhibit uniform size, shape and charge, and carry a known number of primary amine groups on the surface of the molecule, all of which may be used for conjugation purposes. Polyamidoamine dendrimers also bear tertiary amine groups which will be protonated in aqueous solution at physiological pH, conferring aqueous solubility on the carrier molecule.

A polypeptide carrier can be also used instead of aminodextran or a polyamidoamine dendrimer, but the polypeptide carrier must have at least 50 amino acid residues in the chain, preferably 100-5000 amino acid residues. At least some of the amino acids should be lysine residues or glutamate or aspartate residues. The pendant amines of lysine residues and pendant carboxylates of glutamine and aspartate are convenient for attaching a drug, toxin, chelator, or boron addend. Examples of suitable polypeptide carriers include polylysine, polyglutamic acid, polyaspartic acid, co-polymers thereof, and mixed polymers of these amino acids and others, e.g., serines, to confer desirable solubility properties on the resultant loaded carrier and immunoconjugate.

Conjugation of the intermediate conjugate with the antibody component is effected by oxidizing the carbohydrate portion of the antibody component and reacting the resulting aldehyde (and ketone) carbonyls with amine groups remaining on the carrier after loading with a drug, toxin, chelator, boron addend, or label.

Alternatively, an intermediate conjugate can be attached to an oxidized antibody component via amine groups that have been introduced in the intermediate conjugate after loading with the diagnostic or therapeutic principle. Oxidation is conveniently effected either chemically, e.g., with $NaIO_4$ or other glycolytic reagent, or enzymatically, e.g., with neuraminidase and galactose oxidase. In the case of an aminodextran carrier, not all of the amines of the aminodextran are typically used for loading a diagnostic or therapeutic principle. The remaining amines of aminodextran condense with the oxidized antibody component to form Schiff base adducts, which are then reductively stabilized, normally with a borohydride reducing agent.

Analogous procedures are used to produce other immunoconjugates according to the invention. The stoichiometry between the carrier molecule and the diagnostic or therapeutic principle is adjusted such that loaded dendrimer and polypeptide carriers preferably have free amine residues remaining for condensation with the oxidized carbohydrate portion of an antibody component. Carboxyls on the polypeptide carrier can, if necessary, be converted to amines by, e.g., activation with DCC and reaction with an excess of a diamine.

The final immunoconjugate is purified using conventional techniques, such as size-exclusion chromatography on Sephacryl S-300 or similar matrices.

Indirect conjugation to an antibody fragment is illustrated in Example 4.

(ii) Direct conjugation

Alternatively, immunoconjugates can be prepared by directly conjugating an antibody component with a diagnostic or therapeutic principle. The general procedure is analogous to the indirect method of conjugation except that a diagnostic or therapeutic principle is directly attached to an oxidized antibody component. The direct conjugation of chelators to an

antibody fragment is illustrated in Example 3. A particular advantage of preparing immunoconjugates via coupling to the oxidized light chain carbohydrate is that the oxidation reaction provides multiple sites for attachment of diagnostic or therapeutic principles. Since the light chain carbohydrate moiety does not impinge on the antigen binding site, this method therefore provides a means of directly attaching multiple diagnostic or therapeutic principles to an antibody fragment, without the use of a polymeric carrier, to increase antibody loading capacity. This is advantageous in circumstances where the presence of a charged intermediate carrier molecule is associated with unfavorable pharmacokinetics of the immunoconjugate.

It will be appreciated that other diagnostic or therapeutic principles can be substituted for the chelators described below. Those of skill in the art will be able to devise conjugation schemes without undue experimentation.

In addition, those of skill in the art will recognize numerous possible variations of the conjugation methods. In one example, the carbohydrate moiety can be used to attach polyethyleneglycol (PEG) in order to alter the pharmacokinetic properties of an intact antibody, or antigen-binding fragment thereof, in blood, lymph, or other extracellular fluids. This is particularly advantageous for the use of antibody fragments labeled with radiometals, and in particular ^{99m}Tc, in radioimmunodiagnosis (RAID).

^{99m}Tc is a particularly attractive radioisotope for therapeutic and diagnostic applications, as it is readily available to all nuclear medicine departments, is inexpensive, gives minimal patient radiation doses, and has ideal nuclear imaging properties. It has a half-life of six hours which means that rapid targeting of a technetium-labeled antibody is desirable. Consequently, antibody fragments such as $F(ab')_2$ and $F(ab)_2$, and especially Fab and Fab', which show more rapid targeting

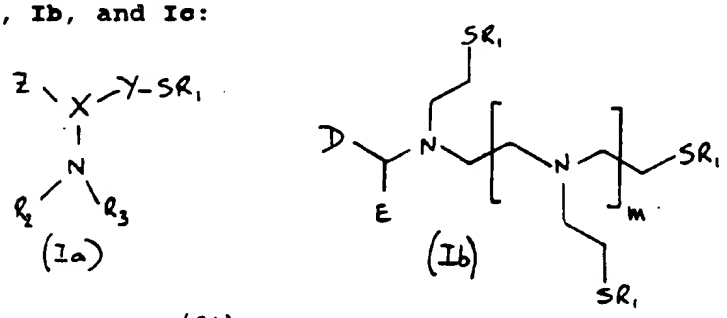
kinetics than whole immunoglobulin, are preferred for RAID applications with Tc-99m labeling. A major drawback to the use of Tc-99m-labeled fragments for imaging is the relatively high uptake and retention of radioactivity in the kidney, which leads to imaging difficulties in the area of this organ. It has been found that conjugation of PEG to Tc-99m-labeled antibody fragments causes a pronounced decrease in the amount of renal uptake and retention of the fragments. See U.S. Patent Application No. 08/309,319, which is herein incorporated by reference in its entirety.

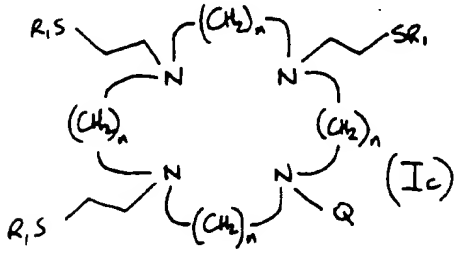
To couple PEG to light chain carbohydrate, the carbohydrate moiety can be oxidized with periodate and coupled with a PEG derivative bearing a nucleophilic moiety by methods well known in the art. For example, PEG hydrazide (Shearwater Polymers, Inc., Huntsville, AL) is mixed with the antibody fragment to form a hydrazone. Alternatively a PEG-amine can be reacted with the oxidized carbohydrate to form a Schiff's base, which is then reduced by treatment with sodium cyanoborohydride to form a stable secondary amine linkage. Conjugation of PEG to a Fab antibody fragment is illustrated in Example 8.

In a preferred embodiment, once the antibody fragment has been conjugated to PEG it can be treated with a reducing agent under controlled conditions to produce free thiol groups which allow direct labeling of the fragment with Tc-99m. Methods for the controlled reduction of antibody fragments are well known to those of ordinary skill in the art. See, for example, U.S. Patent 5,128,119 which is hereby incorporated by reference in its entirety. In another preferred embodiment free thiol groups can be generated on the PEG-conjugated antibody fragment in a non-site specific manner by reaction with a thiolating agent such as Traut's reagent, or as described in U.S Patent Application No. 08/253,772, followed by direct labeling with Tc-99m.

In another embodiment, amine-terminating bifunctional chelating reagents (BFC) are linked to the oxidized light chain carbohydrate of the antibody or antibody fragment. These bifunctional reagents contain pendant thiol and amine groups which are suitably disposed to tightly bind radioactive metals such as ^{186}Re, ^{188}Re, ^{111}Ag, and ^{67}Cu. Conjugation of the BFC to the antibody is achieved through amine or hydrazine functions on the BFC, which can respectively form imine or hydrazone linkages to the aldehyde functions on the oxidized carbohydrate. Imine linkages can be stabilized by reduction with a reducing agent such as sodium cyanoborohydride. During the conjugation step the thiol group of the chelator moiety is masked as a thiol ester or disulfide, and is deprotected after the preparation of the conjugate.

The BFCs can be described by the general structures Ia, Ib, and Ic:





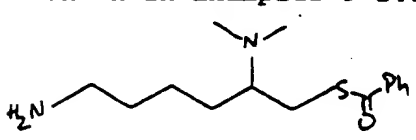
In general structure Ia, X is CH, or X and Z taken together can be CO; Y is CR_4R_5, $CH_2CR_4R_5$ or $(CH_2)_2CR_4R_5$ where R_4 and R_5 are the same or different and are selected from the group consisting of hydrogen and alkyl, substituted alkyl, aryl or substituted aryl groups; Z can be any group capable of reacting and/or complexing with the oxidized carbohydrate groups on the protein, or Z can be

H; R_1 is a thiol protecting group which can be removed under conditions which do not significantly diminish the immunoreactivity of the protein; R_2 and R_3 can be the same or different, and each represent an acyl group or a substituted acyl group, or hydrogen, alkyl, aryl, substituted alkyl, or substituted aryl, where the substituents on the alkyl or aryl groups are metal-ligating groups selected from the group consisting of sulfhydryl, amine and carboxylic acid or their protected derivatives; R_2 and R_3 also can be any group capable of reacting and/or complexing with the oxidized carbohydrate groups on the protein.

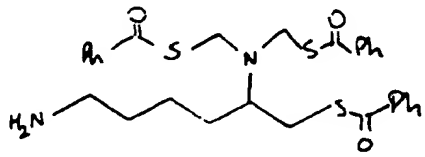
In formula (II) D is H or CH_2SR_1; E can be any group capable of reacting and/or complexing with the oxidized carbohydrate groups on the protein; R_1 is a thiol protecting group which can be removed under conditions which do not significantly diminish the immunoreactivity of the protein, and m is 0, 1, 2, or 3.

In formula (III) Q can be any group capable of reacting and/or complexing with the oxidized carbohydrate groups on the protein; R_1 is a thiol protecting group which can be removed under conditions which do not significantly diminish the immunoreactivity of the protein; and each n independently is 2 or 3.

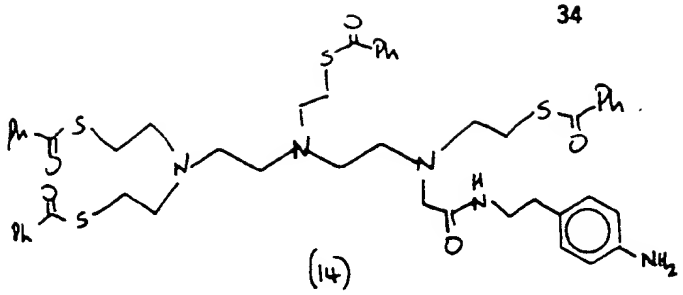
Representative examples of Ia, Ib, and Ic are shown below. In some of these, the antibody-binding group is shown as, but not limited to, a hydrazide. Only the thiol-protected versions of the structures are shown (with 'R' being acyl, benzoyl or 2-thiopyridyl), although metal-complexation will involve thiol-deprotected conjugates. The synthesis of the BFCs can be achieved by methods that are well known in the art. Representative syntheses of some BFCs and methods of conjugation are shown in Examples 9-14.



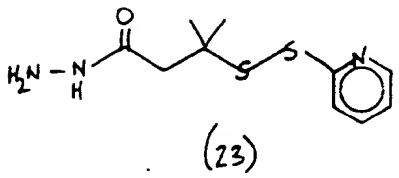
(5)



(10)



(14)



(23)

The thiol protecting group used in the BFC can be any organic or inorganic group which is readily removed under mild conditions to regenerate the free sulfhydryl in the presence of the protein without substantially altering the activity of the protein. Examples of suitable protecting groups include thiol esters, thiocarbamates and disulfides. In a preferred embodiment the thiol protecting group is a benzoate thioester. Those skilled in the art are familiar with the procedures of protecting and deprotecting thiol groups. For example, benzoate thioesters may be deprotected under mild and selective conditions using hydroxylamine. However, when the amine is a hydrazide, the thiol group is most preferably protected as a disulfide, for example with the 'R' function as a 2-pyridylthio group.

In another embodiment of the invention, the oxidized carbohydrate can be used to conjugate groups for pretargeting of the antibody. The pretargeting of monoclonal antibodies is useful for "decoupling" the antibody targeting step and the radiodiagnostic/radiotherapeutic delivery step in antibody-based agents. By reducing the amount of radioisotope in circulation, while maintaining a high uptake of antibody at its target, a reduction in radiation dose to blood and blood-forming tissues, and higher target:non-target ratios of radioisotope are possible. Typical examples of the pretargeting approach are: the use of antibody-avidin (or antibody-streptavidin) conjugates in a prelocalization step, followed by delivery of an isotope conjugated to a biotin moiety; the use of antibody-biotin conjugates in a

prelocalization step followed by delivery of an isotope conjugated to an avidin (or streptavidin) moiety; or the use of antibody-biotin conjugates in a prelocalization step followed by delivery of an avidin (streptavidin) moiety and subsequent delivery of an isotope conjugated to a biotin moiety. Other pairs of agents which may find similar use as secondary targeting vectors are, for example: two complimentary sequences of single-stranded nucleic acids; an enzyme together with its specific substrate; or a protein together with its specific ligand, such as intrinsic factor and vitamin B_{12}.

Additional targeting steps are also feasible and the use of more than one radiolabeled species are also possible as described in U.S. Patent Application No. 08/051,144 which is herein incorporated by reference in its entirety. Other approaches to achieving a higher amount of therapeutic at the antibody target site include the incorporation of, for example, an antibody-biotin(avidin)-radioisotope conjugate as a later-step isotope delivery vehicle, directed to a target pretargeted with antibody-avidin(biotin). In this example the antibody-biotin(avidin)-radioisotope conjugate has two sites, *i.e.* antigen and avidin(biotin), which can be targeted. See, for example, U.S Patent Application No. 08/062,662 which is hereby incorporated by reference in its entirety.

The presence of light-chain carbohydrate on antibody fragments allows for site-specificity of conjugation of suitable pretargeting reagents to antibody fragments such as $F(ab')_2$. Additionally, in the case of Fab' fragments bearing free thiol groups the presence of both the carbohydrate and thiol functions allows site-specific conjugation of two different moieties, each of which can have distinct chemical properties.

Examples of schemes for preparing pretargeting conjugates (underlined) are shown below:

(Avidin-thiol) plus (maleimide-L-hydrazide) forms (Avidin-L-hydrazide)

2(Avidin-L-hydrazide) plus (CHO-Fab'-S-S-Fab'-CHO) forms <u>(Avidin)$_2$-F(ab')$_2$</u>

(Avidin)$_2$-F(ab')$_2$ reduced with 2-mercaptoethanol forms <u>2x Avidin-Fab'-SH</u>

Avidin plus (succinimide-L-maleimide) forms (avidin-L-maleimide)

(Avidin-L-maleimide) plus (Avidin-Fab'-SH) forms <u>(Avidin)$_2$-Fab'</u>

(Avidin-CHO) plus (hydrazide-L-hydrazide) forms (Avidin-L-hydrazide)

2(Avidin-L-hydrazide) plus (CHO-Fab'-S-S-Fab'-CHO) forms <u>(Avidin)$_2$-F(ab')$_2$</u>

(Avidin)$_2$-F(ab')$_2$ reduced with 2-mercaptoethanol forms <u>2(Avidin-Fab'-SH)</u>

Avidin-Fab'-SH plus (Avidin-maleimide) forms <u>(Avidin)$_2$-Fab'</u>

n(Biotin-L-hydrazide) plus (CHO-Fab'-S-S-Fab'-CHO) forms <u>(Biotin)$_n$-F(ab')$_2$</u>

where n is an integer, usually from 1 to about 30; L designates a linker, hydrocarbon, alkyl, acyl or a combination which separates two distinct reactive functionalities, and which encompasses commercially available protein cross-linking agents. Streptavidin may be used in place of avidin in the examples described above. Carbohydrate moieties can be oxidized to produce aldehydes and disulfide bonds reduced to generate free thiols, when indicated, using standard reagents such as sodium periodate and 2-mercaptoethanol, respectively. Thiol groups may be introduced onto avidin by use of known thiolating agents such as 2-iminothiolane. Free thiol groups on the avidin(streptavidin)-Fab' conjugates optionally may be blocked, for instance with

iodoacetamide, prior to their use. Alternatively, the free thiol group may be used as a reactive group for further modification, for example by radiolabeling with Tc-99m, or by conjugation with an agent such as a poly(ethylene glycol) (PEG) derivative activated via a maleimide reaction for subsequent coupling to free thiol groups.

The $F(ab')_2$-based streptavidin/avidin conjugates retain two non-sterically compromised antigen-binding sites and all eight biotin-binding sites, and monovalent Fab' units carry one or two streptavidin/avidin units per Fab' with full retention of biotin-binding ability. Use of the carbohydrate means that several biotin units can be coupled to each fragment molecule via the oxidized carbohydrate without interfering with the fragment's antigen-binding capability.

In another embodiment of the invention, conjugation to light-chain carbohydrate residues that are distant from the antigen binding site ensures that interference of binding of a subsequently-administered clearing second antibody will not take place if the second antibody is an antiidiotypic antibody. In this instance, the second antibody will bind to the circulating antibody through its antigen binding site, and the targeting antibody will clear *via* the liver. Use of this system has the advantage that none of the targeting antibody's secondary sites (*e.g.* avidins or biotins) are blocked during the clearing step.

In another embodiment of the invention, chelates bearing radioactive nuclides can be linked to the oxidized light-chain carbohydrate *via* metabolizable linkages. A problem frequently encountered with the use of antibody fragments in radiotherapeutic and radiodiagnostic applications is a potentially dangerous accumulation of the radiolabeled antibody fragments in the kidney. When the conjugate is formed using a acid-or base-labile linker, cleavage of the radioactive chelate from the antibody can advantageously occur. If the

chelate is of relatively low molecular weight, it is not retained in the kidney and is excreted in the urine, thereby reducing the exposure of the kidney to radioactivity.

Low molecular weight chelates suitable for this application include, for example, the bifunctional chelates described above, and DOTA or DTPA-type chelates. Each of these molecules can be modified, by standard methods known in the art, to provide reactive functional groups which can form acid-labile linkages with carbonyl groups on the oxidized carbohydrate of the antibody fragment. Examples of suitable acid-labile linkages include hydrazone and thiosemicarbazone functions. These are formed by reacting the oxidized carbohydrate with chelates bearing hydrazide, thiosemicarbazide, and thiocarbazide functions, respectively. The preparation and conjugation of a thiocarbazide derivative of DTPA is demonstrated in Example 15.

Alternatively, base-cleavable linkers, which have been used for the enhanced clearance of bifunctional chelate-^{99m}Tc-labeled fragments from the kidneys, can be used. See, for example, Weber *et al.* Bioconjug. Chem. 1:431 (1990). The coupling of a bifunctional chelate to light-chain carbohydrate *via* a hydrazide linkage can incorporate base-sensitive ester moieties in a linker spacer arm. Such an ester-containing linker unit is exemplified by ethylene glycolbis(succinimidyl succinate), (EGS, available from Pierce Chemical Co., Rockford, IL), which has two terminal N-hydroxysuccinimide (NHS) ester derivatives of two 1,4-dibutyric acid units, each of which are linked to a single ethylene glycol moiety by two alkyl esters. One NHS ester may be replaced with a suitable amine-containing BFC (for example 2-aminobenzyl DTPA), while the other NHS ester is reacted with a limiting amount of hydrazine. The resulting hyrazide is used for coupling to the light-chain carbohydrate of an antibody or antibody fragment, forming an antibody-BFC linkage

containing two alkyl ester functions. Such a conjugate is stable at physiological pH, but readily cleaved at basic pH.

In another embodiment of the invention, it is possible to construct a "divalent immunoconjugate" by attaching a diagnostic or therapeutic principle to a carbohydrate moiety and to a free sulfhydryl group. Such a free sulfhydryl group may be located in the hinge region of the antibody component.

6. Use of Immunoconjugates for Diagnosis and Therapy

A. Use of Immunoconjugates for Diagnosis

The method of diagnostic imaging with radiolabeled monoclonal antibodies is well known. See, for example, Srivastava (ed.), RADIOLABELED MONOCLONAL ANTIBODIES FOR IMAGING AND THERAPY, Plenum Press (1988); Chase, "Medical Applications of Radioisotopes," in REMINGTON'S PHARMACEUTICAL SCIENCES, 18th Edition, Gennaro *et al.* (eds.) Mack Publishing Co., pp. 624-652 (1990); and Brown, "Clinical Use of Monoclonal Antibodies," in BIOTECHNOLOGY AND PHARMACY, Pezzuto *et al.* (eds.), Chapman & Hall, pp. 227-249 (1993). This technique, also known as immunoscintigraphy, uses a gamma camera to detect the location of gamma-emitting radioisotopes conjugated to monoclonal antibodies. Diagnostic imaging can be used to diagnose cardiovascular disease and infectious disease. Brown, *supra*.

The present invention contemplates the use of immunoconjugates to diagnose cardiovascular disease. For example, immunoconjugates comprising anti-myosin fragments can be used for imaging myocardial necrosis associated with acute myocardial infarction. Immunoconjugates comprising antibody fragments that bind platelets and fibrin can be used for imaging deep-vein thrombosis. Moreover, immunoconjugates comprising

antibody fragments that bind to activated platelets can be used for imaging atherosclerotic plaque.

Immunoconjugates of the present invention also can be used in the diagnosis of infectious diseases. For example, immunoconjugates comprising antibody fragments that bind specific bacterial antigens can be used to localize abscesses. In addition, immunoconjugates comprising antibody fragments that bind granulocytes and inflammatory leukocytes can be used to localize sites of bacterial infection.

Numerous studies have evaluated the use of monoclonal antibodies for scintigraphic detection of cancer. See, for example, Brown, *supra*, and references therein. Investigations have covered the major types of solid tumors such as melanoma, colorectal carcinoma, ovarian carcinoma, breast carcinoma, sarcoma, and lung carcinoma. Thus, the present invention contemplates the detection of cancer using immunoconjugates comprising antibody fragments that bind tumor markers to detect cancer. Examples of such tumor markers include carcinoembryonic antigen, alpha-fetoprotein, oncogene products, tumor-associated cell surface antigens, and necrosis-associated intracellular antigens.

In addition to diagnosis, monoclonal antibody imaging can be used to monitor therapeutic responses, detect recurrences of a disease, and guide subsequent clinical decisions.

For diagnostic imaging, radioisotopes may be bound to antibody fragments either directly or indirectly by using an intermediary functional group. Such intermediary functional groups include DOTA, DTPA and EDTA. The radiation dose delivered to the patient is maintained at as low a level as possible. This is accomplished through the choice of isotope for the best combination of minimum half-life, minimum retention in the body, and minimum quantity of isotope which will permit detection and accurate measurement. Examples of radioisotopes which can be bound to antibodies and are

appropriate for diagnostic imaging include ^{99m}Tc and ^{111}In.

Studies indicate that antibody fragments, particularly Fab and Fab', provide advantageous tumor/background ratios. Brown, *supra*. Thus, the use of Fab and Fab' antibody fragments for the preparation of immunoconjugates is a preferred embodiment of the invention. However, the retention of divalency when using a $F(ab)_2$ or a $F(ab')_2$ targeting vector leads to higher absolute amounts of antibody at the target compared to monovalent fragments, and can lead to better target:non-target ratios in some tissues.

The immunoconjugates useful in the invention also can be labeled with paramagnetic ions for purposes of *in vivo* diagnosis. Elements which are particularly useful for magnetic resonance imaging include Gd^{III}, Mn, Dy, and Fe ions.

In one embodiment of the invention, multiple chelate molecules, such as DTPA, are directly conjugated to the oxidized light chain carbohydrate of the antibody fragment, allowing the chelation of a large number of paramagnetic ions without the need for an intermediate carrier. The use of some types of intermediate carrier has been observed to have deleterious effects on the magnetic resonance imaging results achieved with metal chelates. See, for example, Wiener, *et al.*, *Magnetic Resonance in Medicine* 31:1-8 (1994). Direct conjugation of chelates in this way therefore eliminates such problems.

In another embodiment of the invention, the immunoconjugate uses a polyamidoamine dendrimer as an intermediate carrier for attachment of a chelating moiety such as DTPA. Such dendrimers have been shown to possess several advantages over other molecules for use as carriers of paramagnetic ions for magnetic resonance imaging. See, for example, Wiener, *et al.*, *supra*.

The present invention also contemplates the use of immunoconjugates to detect the presence of particular antigens *in vitro*. In such immunoassays, the